Regulatory Toxicology and Pharmacology 70 (2014) S26–S40

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Toxicological assessment of kretek cigarettes part 3: Kretek and American-blended cigarettes, inhalation toxicity

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ARTICLE INFO

Article history:
Available online 22 October 2014

Keywords:
Kretek
Smoke
Eugenol
Clove
Rat
Inhalation

ABSTRACT

A typical Indonesian kretek cigarette brand and an experimental kretek reference cigarette were compared to the reference cigarette 2R4F in two 90-day inhalation studies. Male and female rats were exposed nose-only to mainstream smoke for 6 hours daily, for 90 consecutive days. Biological endpoints were assessed according to OECD guideline 413, with special emphasis on respiratory tract histopathology and on lung inflammation (broncho-alveolar lavage fluid levels of neutrophils, macrophages and lymphocytes). Histopathological alterations included: in the nose, hyperplasia and squamous metaplasia of the respiratory epithelium and squamous metaplasia and atrophy of the olfactory epithelium; in the larynx, epithelial squamous metaplasia and hyperplasia; in the lungs, accumulation of macrophages in alveoli and goblet cell hyperplasia in bronchial epithelium. The findings were qualitatively consistent with observations from previous similar studies on conventional cigarettes. Compared to 2R4F cigarette, however, kretek smoke exposure was associated with a pronounced attenuation of pulmonary inflammation and less severe histopathological changes in the respiratory tract. Neutrophilic inflammation was also significantly lower (>70%). These results are consistent with the observations made on smoke chemistry and *in vitro* toxicology. They do not support any increased toxicity of the smoke of kretek cigarettes compared to conventional American-blended cigarettes.

1. Introduction

This publication is part of a series summarizing the *in vitro* and *in vivo* toxicological assessment of kretek cigarettes. Smoke composition and biological activity of mainstream smoke (MS) from marketed and experimental kretek cigarettes were evaluated on a comparative basis in smoke chemistry analyses, and *in vitro* and *in vivo* toxicity studies. The studies were designed to cover three main topics: (1) characterization of kreteks and comparison relative to American-blended cigarettes, (2) impact of blend type and cloves, and (3) impact of ingredients used in kretek cigarettes. Further in depth information of this assessment is described in the lead publication (Roemer et al., 2014b).

In Part 2 of the current series of publications, both smoke composition and biological activity of MS from two marketed kretek cigarettes, including the *Gudang Garam International Filter* (hereafter abbreviated as Garam), and a kretek reference (Kretek-R) cigarette were evaluated relative to the reference cigarette 2R4F by smoke chemistry analyses and *in vitro* toxicity studies (Piadé et al., 2014). In this part the *in vivo* results of two independent 90-day inhalation studies, in which Garam and Kretek-R cigarettes were benchmarked against the reference cigarette 2R4F, are presented.

2. Materials and methods

2.1. General

Garam and Kretek-R cigarettes were evaluated against the American-blended reference cigarette 2R4F in two separate 90-day inhalation studies (Study A and B, respectively) conducted in compliance with the Organization for Economic Co-operation and Development (OECD) Principles on Good Laboratory Practice (GLP) (as revised in 1997) and according to the OECD guideline 413 (OECD, 1981).

The inhalation studies were conducted in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care

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http://dx.doi.org/10.1016/j.yrtph.2014.09.013
0273-2300/

International)-accredited facility (AAALAC, 2006) in Leuven, Belgium (Philip Morris Research Laboratories) where the care and use of rats was conformed to the American Association for Laboratory Animal Science Policy on the Humane Care and Use of Laboratory Animals (www.aalas.org). The studies were approved by the local Institutional Animal Care and Use Committee according to Belgian legislation.

Detailed descriptions regarding the chemical analyses, toxicological assays and statistical procedures can be found in the first of this series of publications (Roemer et al., 2014b). The smoke generation, the analytical characterization of the test atmosphere, the selection and care of animals and the selection of exposure parameters were performed as previously detailed (Vanscheeuwijck et al., 2002). They can be summarized as follows:

2.2. Cigarettes

The cigarettes tested were a commercial Indonesian kretek cigarette, the *Gudang Garam International Filter* (abbreviated Garam) and an experimental kretek reference cigarette (Kretek-R) designed to be representative of an average kretek cigarette. A detailed description of the design and performance of both cigarettes can be found in Part 2 of this publications series (Piadé et al., 2014).

2.3. Smoke generation

A rotary smoking machine, operated according to International Organization for Standardization (ISO) 3308 conditions (ISO-3308, 2000) was used to continuously generate MS following ISO 4387 standard requirements (ISO-4387-4, 2000). Smoke was diluted at machine exit with filtered and conditioned air to match the target concentration of total particulate matter (TPM) and fed to the exposure chambers.

2.4. Exposure

Both inhalation studies were performed using TPM target concentrations of 100 μg/l (Low), 150 μg/l (Medium) and 200 μg/l (High) for both kretek cigarettes as well as for the reference cigarette 2R4F. Outbred male and female Sprague–Dawley rats were exposed nose-only for 6 hours daily, for 90 consecutive days. A control group of animals was exposed to filtered, conditioned air (Sham). The study was performed with 10 rats per group, per sex and per necropsy time point. The mean temperature of the test atmosphere was 21.6 °C and relative humidity 54%. The inhalation period began with a dose adaptation period (i.e., ¼, ½, and ¾ of the final daily exposure duration on days 1, 2, and 3, respectively). An interim assessment was conducted for all end points after 35 days of exposure, and post-inhalation animals were allowed a 42-day recovery before sacrifice to investigate reversibility, persistence, and delayed occurrence of smoke-related effects.

To characterize the test atmosphere and check the reproducibility of MS generation and dilution, the concentration of TPM and carbon monoxide (CO) was determined daily. In addition the airborne concentrations of acetaldehyde, acrolein, formaldehyde and nicotine were determined weekly, and the particle size distribution was assessed on two different occasions.

2.5. Endpoints

General health of animals was monitored by body weight and food consumption. The endpoints analyzed included all parameters specified in the OECD guideline 413 (OECD, 1981), and were determined at the interim sacrifice (after 35 days) and at the end of the inhalation period.

Weight was determined for major organs. Lung inflammatory response was assessed by quantification of neutrophils, lymphocytes, and alveolar macrophages in broncho-alveolar lavage fluid (BALF) (Friedrichs et al., 2006). Histopathological examination was performed with an extended assessment of larynx, tracheal bifurcation, and lung.

3. Results

The observations made after the 35-day interim period were in line with those made at the end of the 90-day exposure period, and the following discussion is thus based on the latter. Data are however included in the atmosphere characterization or the animals body weight development.

3.1. Test atmospheres characterization

The concentrations of both TPM and the monitored smoke constituents were found to be stable throughout the 90-day exposure period, and target TPM concentrations were achieved (Table 1).

Table 1
Test atmosphere in exposure chambers for the two separate 90-day inhalation studies (Study A and B).

Group	TPM (μg/l)	Particle size		Nicotine (μg/l)	Carbon monoxide (ppm)	Acetaldehyde (μg/l)	Acrolein (μg/l)	Formaldehyde (μg/l)
		MMAD (μm)	GSD					
Study A								
Sham	<0.9	–	–	<0.03	<1.5	–	–	–
2R4F Low	99.9 ± 4.9	0.52	1.67	5.43 ± 0.32	114.3 ± 5.6	4.61 ± 0.38	0.35 ± 0.03	0.13 ± 0.01
2R4F Medium	149.8 ± 7.2	0.52	1.66	8.14 ± 0.91	165.9 ± 8.1	6.61 ± 0.51	0.52 ± 0.04	0.19 ± 0.03
2R4F High	204.6 ± 9.0	0.53	1.69	12.03 ± 1.10	227.5 ± 9.2	8.57 ± 1.46	0.65 ± 0.11	0.23 ± 0.04
Garam Low	99.6 ± 7.7	0.56	1.70	5.30 ± 0.68	48.6 ± 3.7	1.62 ± 0.13	0.16 ± 0.02	0.21 ± 0.02
Garam Medium	145.5 ± 10.5	0.58	1.70	7.20 ± 0.82	68.4 ± 3.8	2.25 ± 0.34	0.22 ± 0.03	0.29 ± 0.04
Garam High	200.7 ± 12.6	0.58	1.72	8.89 ± 1.27	88.3 ± 5.6	2.89 ± 0.28	0.30 ± 0.03	0.35 ± 0.04
Study B								
Sham	<1.6	–	–	<0.03	<4.5	–	–	–
2R4F Low	98.0 ± 3.9	0.69	1.72	7.65 ± 1.02	112.0 ± 5.8	6.69 ± 0.32	0.72 ± 0.04	0.13 ± 0.01
2R4F Medium	149.3 ± 6.1	0.60	1.67	11.53 ± 1.00	168.5 ± 9.3	9.90 ± 0.48	1.10 ± 0.10	0.19 ± 0.02
2R4F High	197.6 ± 10.0	0.60	1.70	15.24 ± 0.62	208.4 ± 13.3	12.90 ± 0.68	1.40 ± 0.08	0.24 ± 0.02
Kretek-R Low	98.1 ± 5.9	0.65	1.80	6.84 ± 0.72	62.7 ± 3.9	3.27 ± 0.16	0.46 ± 0.02	0.23 ± 0.02
Kretek-R Medium	147.8 ± 6.0	0.68	1.69	9.58 ± 0.82	90.3 ± 3.4	4.69 ± 0.10	0.66 ± 0.02	0.33 ± 0.02
Kretek-R High	198.6 ± 9.1	0.68	1.73	12.01 ± 1.07	117.1 ± 5.0	5.73 ± 0.62	0.80 ± 0.09	0.42 ± 0.03

Results represent medians or means ± standard deviation.
Abbreviations: MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation; Garam, *Gudang Garam International Filter*; Kretek-R, *kretek reference cigarette;* TPM, total particulate matter.

Particle size distribution measurements, i.e., mass median aerodynamic diameter (MMAD) and its geometric standard deviation (GSD), indicated that particles were equally respirable in all smoke-exposed groups.

At the lowest target concentration of TPM (100 μg/l) the nicotine concentrations in the test atmospheres were similar for Garam and the reference cigarette 2R4F, but 10% lower for Kretek-R cigarette. Compared to the medium and high TPM target concentrations for the reference cigarette 2R4F, nicotine concentrations in the exposure atmospheres were approximately 10% and 25% lower for the Garam and Kretek-R cigarettes, respectively. With regards to the other smoke constituents monitored in the exposure chambers, concentrations of CO, acrolein and acetaldehyde were in all cases substantially lower in the test atmospheres generated from kretek cigarettes than in those generated from the reference cigarette 2R4F at similar target concentration TPM levels. In contrast, the concentration of formaldehyde was higher, which is in line with the cigarette smoke chemical composition data (Piadé et al., 2014).

3.2. Clinical observations

After daily exposure, Harderian gland secretion and wet fur were observed in sham- and smoke-exposed groups; incidences in the smoke-exposed groups were higher than in the sham group. In some cases, increased reaction to sound and touch, decreased turning reflex, and decreased gripping ability were observed. Such observations have also been made in previous cigarette smoke inhalation studies (Vanscheeuwijck et al., 2002) and are considered to be related to tube restraint and/or smoke exposure. There were no numerical differences in clinical observations or mortality between the Garam or Kretek-R and the reference cigarette 2R4F groups that could be considered to indicate an influence of the cigarette type.

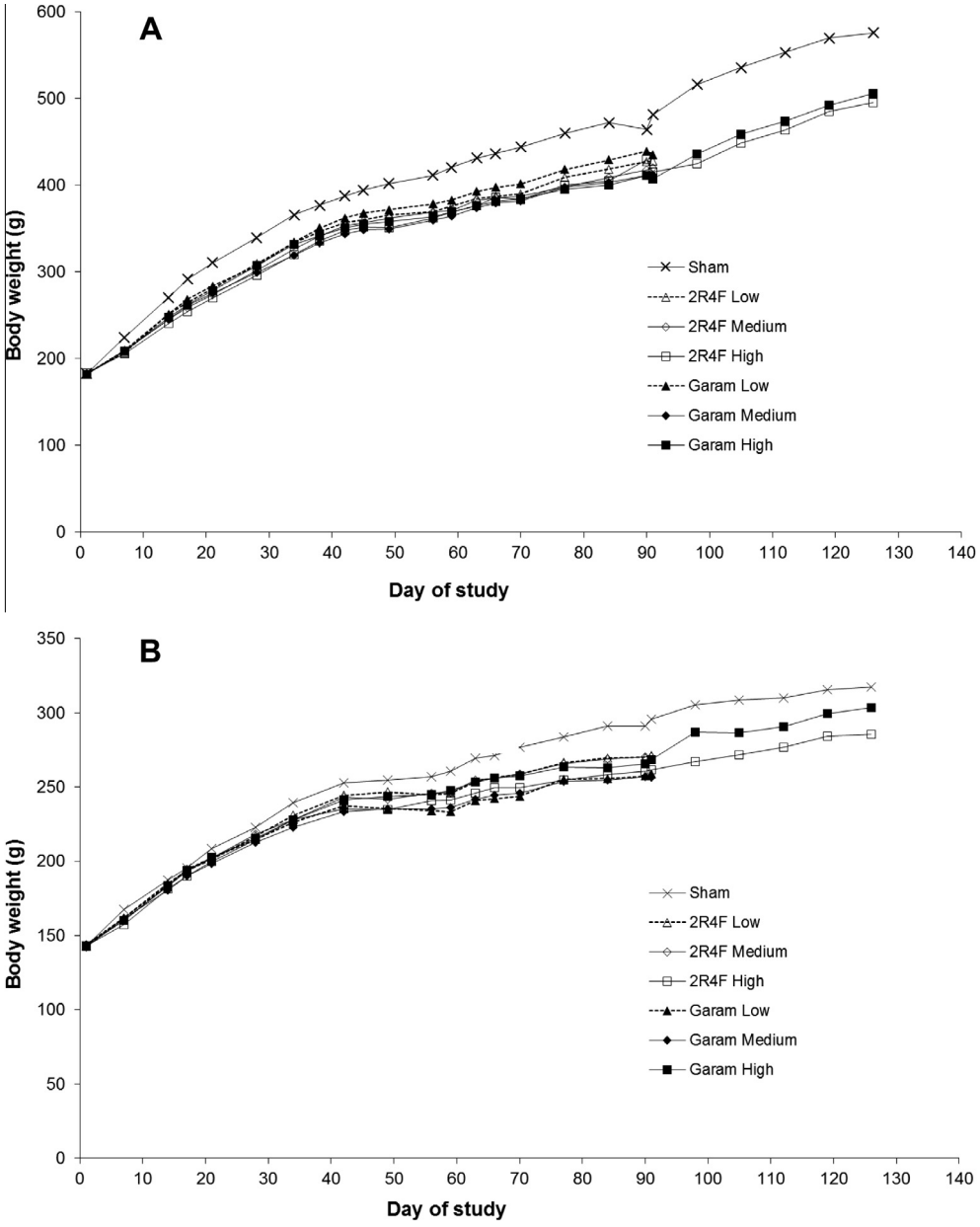

Fig. 1. Mean body weights of male (A) and female (B) rats exposed to fresh air (sham) and smoke of the Garam and the reference 2R4F cigarettes during the 90-day inhalation study (Study A).

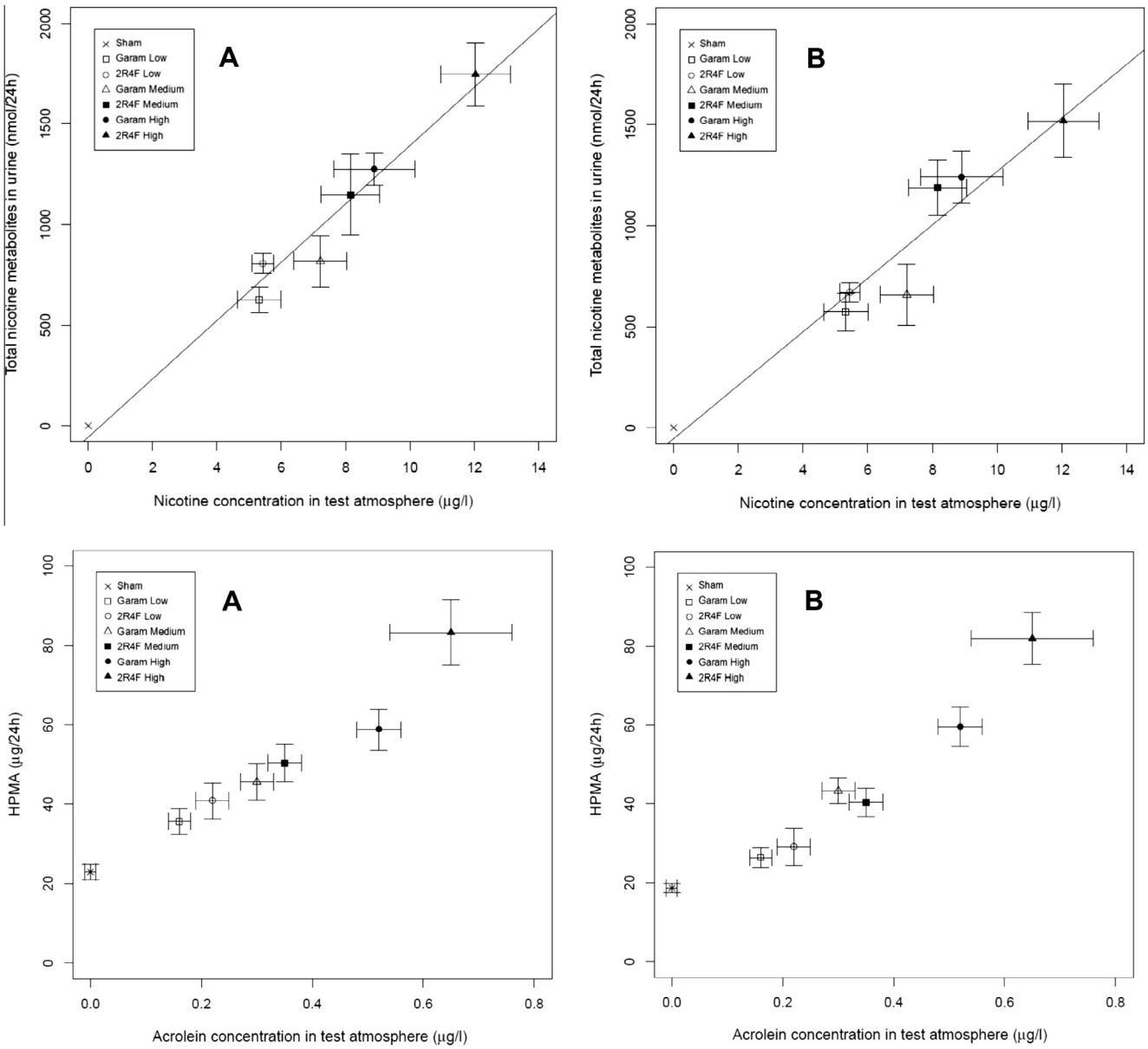

Fig. 2. Total nicotine metabolites and 3-hydroxypropylmercapturic acid (HPMA) levels in 24-h urine samples from male (A) and female (B) rats as a function of exposure chamber precursor concentration (Study A) Note: no regression line is given in the HPMA vs. acrolein plot because the relationship of the urinary biomarker level to acrolein exposure may not be linear (Zheng et al., 2013), and because other sources coexist for HPMA, as demonstrated by the non-zero sham level.

3.3. Body weight

Rats gained weight throughout the studies in all groups, although animals exposed to smoke had reduced body weight relative to sham-treated animals. After the 90-day exposure to cigarette smoke, body weight was about 10–15% lower than sham for male animals and approximately 8–13% lower than sham for female animals. As shown in Fig. 1, no significant difference in animals body weight was observed throughout the 90-day inhalation period whether animals were exposed to smoke from the reference cigarette 2R4F or the Garam cigarette (Study A). The same was observed in Study B with the Kretek-R (data not shown). After the post-inhalation period, however, animals that had been exposed to smoke from Garam cigarettes generally had slightly higher body weights than animals exposed to the smoke of the reference cigarette 2R4F. This trend was more important in the case of female animals and was observed in both studies A and B.

3.4. Respiratory physiology

In general, respiratory frequency, tidal volume, and respiratory minute volume were lower in smoke-exposed groups than in the sham group. There were sporadic statistically significant differences between the various groups, but no overall trends were noted (data not shown).

3.5. Urinary nicotine metabolites

Urinary nicotine metabolites were determined according to Rustemeier et al. (1993). The relative abundances of the measured urinary nicotine metabolites (nicotine-N′-oxide, nornicotine, cotinine, 3′-hydroxycotinine, 5′-hydroxycotinine, norcotinine, 4-(3-pyridyl)-4-oxobutyric acid, 4-(3-pyridyl)-4-hydroxybutyric acid, and 3-pyridylacetic acid) in 24-hour samples were similar in all smoke-exposed groups within each gender. The total amount

Table 2
Blood neutrophil counts after 90-day inhalation exposure.

Group	Neutrophils ($\times 10^9$/l)	
	Male	Female
Study A		
Sham	0.98 ± 0.14	0.71 ± 0.06
2R4F low	1.21 ± 0.21	0.74 ± 0.10
2R4F Medium	1.35 ± 0.21	1.12 ± 0.16
2R4F high	1.69 ± 0.28	1.45 ± 0.18**
Garam low	1.03 ± 0.11	0.55 ± 0.09
Garam medium	0.86 ± 0.13	0.82 ± 0.12
Garam high	1.32 ± 0.18	1.14 ± 0.23
Study B		
Sham	0.66 ± 0.14	0.59 ± 0.07
2R4F low	1.33 ± 0.12*	0.95 ± 0.13
2R4F medium	1.47 ± 0.20**	1.25 ± 0.16*
2R4F high	1.63 ± 0.14***	1.75 ± 0.42**
Kretek-R low	1.00 ± 0.13	0.71 ± 0.13
Kretek-R medium	0.88 ± 0.13	1.19 ± 0.17
Kretek-R high	1.32 ± 0.18*	1.01 ± 0.14

Results are presented as mean ± standard error.
Difference from sham; Significance: *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.
(p-Value from 2-way ANOVA followed by Dunnett posthoc test).
Abbreviations: Garam, *Gudang Garam International Filter;* Kretek-R, *kretek reference cigarette.*

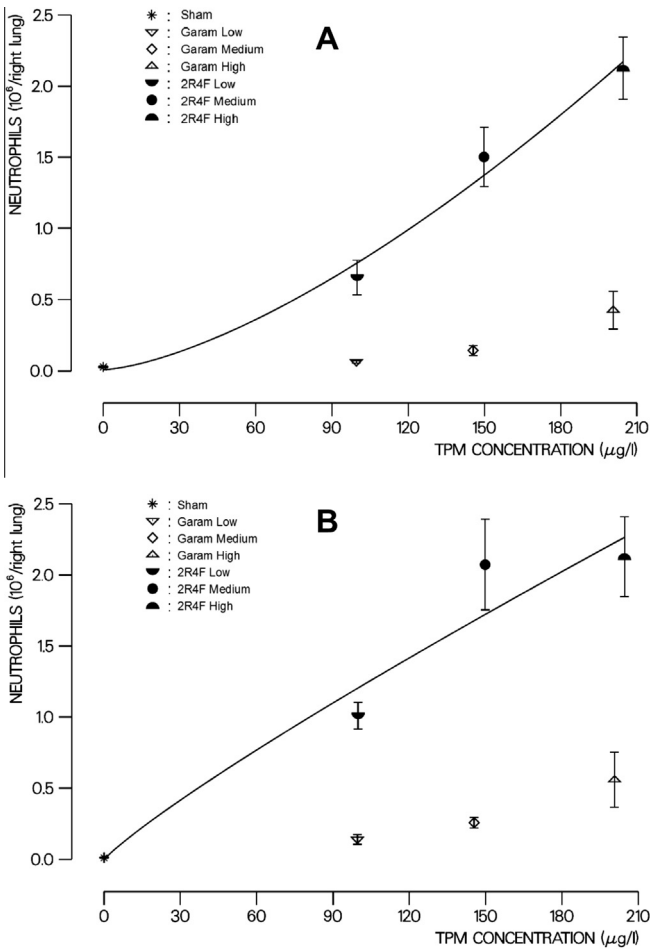

Fig. 3. Neutrophil counts in the BALF from male (A) and female (B) animals exposed to smoke from 2R4F and Garam cigarettes (Study A).

of the urinary metabolites measured in both male and female animals correlated linearly with the nicotine concentration in the test atmosphere, as demonstrated in the case of Garam and the reference cigarette 2R4F in Fig. 2. A similar correlation was also observed between 3-hydroxypropylmercapturic acid (HPMA), biomarker of exposure to acrolein (Carmella et al., 2007), and the acrolein concentration in the test atmosphere (Fig. 2). Similar results were obtained in the case of the Kretek-R cigarette (data not shown).

3.6. Carboxyhemoglobin and other gas phase biomarkers

The steady-state concentrations of carboxyhemoglobin in blood ranged between 12% and 25% in the low and high reference cigarette 2R4F groups, respectively, and between 6% and 13% in the low and high kretek cigarette groups, respectively. These values are consistent with CO concentrations measured in the test atmosphere of the different cigarettes tested. The concentration of 1,3-butadiene was not measured in the test atmosphere, but recovered amounts in 24-hour urine of 3-monohydroxybutylmercapturic acid (MHBMA), a biomarker of exposure to 1,3-butadiene, correlated linearly with test atmosphere concentrations of CO, a good proxy for gases that do not exhibit adsorption to surfaces (data not shown).

3.7. Hematology

At the end of the 90-day exposure period, a statistically significant increase in erythrocyte count, hematocrit, and hemoglobin was observed in the animals of both the reference cigarette 2R4F High and Kretek-R High groups relative to the animals of the sham-exposed group (data not shown). A similar increase was not observed in the animals of the Garam groups and there was no difference between smoke-exposed groups. Compared to sham, the neutrophil count was statistically significantly increased in a dose-dependent manner in animals exposed to MS from the reference cigarette 2R4F, but reached statistical significance only in the Kretek-R High group. The neutrophil count was generally lower in animals exposed to smoke from kretek cigarettes than in those exposed to similar concentrations of TPM from the reference cigarette 2R4F, and the difference reached statistical significance. Data are given in Table 2.

3.8. Clinical chemistry

The observed changes in clinical chemistry parameters are consistent with the observations from previous cigarette smoke inhalation studies (Vanscheeuwijck et al., 2002). These included an increase in the activity of alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase (data not shown). The concentrations of total cholesterol, triglyceride, and total protein were decreased compared to sham, but there were no differences among the smoke-exposed groups.

3.9. Differential cell count in BALF

All smoke-exposed groups had a statistically significant increase in the number of neutrophils in the BALF, compared to sham. This increase was TPM concentration-dependent. The neutrophil counts were statistically significantly lower in the BALF from animals exposed to smoke from Garam and Kretek-R cigarettes than in those exposed to similar TPM levels of smoke from the reference cigarette 2R4F (Fig. 3, Tables 3 and 4). The number of lymphocytes and total amount of cells recovered from BALF were higher in reference cigarette 2R4F-exposed rats than in sham-exposed rats, but this was not the case for kretek cigarette-exposed rats. Relevant BALF cell differential results are summarized in Tables 3 and 4. Table 5 further summarizes all observations for which data were significantly different when groups exposed to

Table 3
Differential cell count in BALF after 90-day inhalation exposure; Garam and reference cigarette 2R4F (Study A).

Cell type	Gender	Sham	2R4F low	2R4F medium	2R4F high	Garam low	Garam medium	Garam high
All cell types	M	7.4 ± 0.6	10.5 ± 0.8*	11.2 ± 0.8**	12.2 ± 1.0***	7.4 ± 0.6	7.4 ± 0.6	7.3 ± 0.8
	F	5.6 ± 0.6	6.1 ± 0.4	8.5 ± 0.7***	7.7 ± 0.6*	4.8 ± 0.3	6.0 ± 0.5	5.6 ± 0.4
Neutrophils	M	0.027 ± 0.007	0.65 ± 0.11***	1.50 ± 0.21 ***	2.13 ± 0.22***	0.062 ± 0.008*	0.14 ± 0.03***	0.43 ± 0.13***
	F	0.011 ± 0.003	1.01 ± 0.09***	2.07 ± 0.32***	2.13 ± 0.28***	0.139 ± 0.033**	0.257 ± 0.038***	0.56 ± 0.19***
Alveolar macrophages	M	7.2 ± 0.6	9.7 ± 0.7	9.6 ± 0.8	9.9 ± 1.1	7.2 ± 0.6	7.2 ± 0.6	6.8 ± 0.8
	F	5.5 ± 0.6	5.0 ± 0.4	6.4 ± 0.6	5.4 ± 0.5	4.6 ± 0.3	5.7 ± 0.5	5.0 ± 0.4
Lymphocytes	M	0.120 ± 0.018	0.157 ± 0.034	0.144 ± 0.014	0.221 ± 0.025**	0.060 ± 0.010	0.054 ± 0.008	0.067 ± 0.011
	F	0.096 ± 0.038	0.103 ± 0.015	0.121 ± 0.018	0.134 ± 0.019	0.066 ± 0.014	0.044 ± 0.007	0.053 ± 0.006

Cell numbers reported as 10^6 cells/right lung. Results are presented as mean ± standard error.
Difference from sham; Significance: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).
Abbreviations: Garam, *Gudang Garam International Filter*; Kretek-R, *kretek reference cigarette*; BALF, broncho-alveolar lavage fluid; M, male; F, female.

Table 4
Differential cell count in BALF after 90-day inhalation exposure; Kretek-R and reference cigarette 2R4F (Study B).

Cell type	Gender	Sham	2R4F low	2R4F medium	2R4F high	Kretek-R low	Kretek-R medium	Kretek-R high
All cell types	M	65.0 ± 5.7	97.0 ± 6.2**	103.8 ± 11.9**	116.5 ± 7.8***	67.3 ± 3.8	80.8 ± 7.1	91.9 ± 6.1*
	F	44.6 ± 4.8	67.1 ± 5.1	107.0 ± 11.4***	116.8 ± 19.5***	66.9 ± 8.9	69.0 ± 8.1	78.4 ± 7.6**
Neutrophils	M	0.70 ± 0.43	8.62 ± 1.70***	16.58 ± 2.59***	27.50 ± 2.80***	1.48 ± 0.30***	2.94 ± 0.38***	3.92 ± 0.50***
	F	0.68 ± 0.56	6.68 ± 0.77***	19.38 ± 3.13***	31.67 ± 3.76***	1.15 ± 0.29***	6.10 ± 1.42***	10.20 ± 1.94***
Alveolar macrophages	M	63.7 ± 5.6	87.1 ± 5.2*	85.8 ± 11.0	87.2 ± 6.9*	65.2 ± 3.5	77.1 ± 6.9	87.1 ± 6.3*
	F	43.5 ± 4.5	59.7 ± 5.3	85.8 ± 10.4*	83.4 ± 16.0*	65.3 ± 8.7	62.3 ± 7.6	67.5 ± 6.9
Lymphocytes	M	0.65 ± 0.16	1.23 ± 0.13**	1.40 ± 0.25***	1.76 ± 0.21***	0.58 ± 0.11	0.71 ± 0.08	0.85 ± 0.17
	F	0.38 ± 0.09	0.71 ± 0.06**	1.72 ± 0.35***	1.76 ± 0.36***	0.46 ± 0.08	0.61 ± 0.08*	0.63 ± 0.06*

Cell numbers reported as 10^6 cells/right lung. Results are presented as mean ± standard error.
Difference from sham; Significance: $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).
Abbreviations: Garam, *Gudang Garam International Filter,* Kretek-R, *kretek reference cigarette*; BALF, broncho-alveolar lavage fluid; M, male; F, female.

Table 5
Significance of differences in BALF cell count after the 90-day inhalation study between animals exposed to MS from reference cigarette 2R4F and those exposed to MS from each of the kretek cigarettes.

Measured parameter	Study A: Garam vs. 2R4F		Study B: Kretek-R vs. 2R4F	
	Male rats	Female rats	Male rats	Female rats
All cell types	***↓	**↓	***↓	**↓
Neutrophils	***↓	***↓	***↓	***↓
Alveolar macrophages	***↓	=	=	=
Lymphocytes	***↓	***↓	***↓	***↓

Difference among cigarettes; significance: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).
Symbols, ↓ indicates response lower in kretek cigarette relative to 2R4F; =indicates no trend.
Abbreviations: Garam, *Gudang Garam International Filter*; Kretek-R, *kretek reference cigarette.*

smoke from either kretek cigarette were compared to groups exposed to equivalent TPM levels of smoke from the reference cigarette 2R4F.

3.10. Non-respiratory tract organs

Minimal to mild thymus atrophy was observed in male and female rats of all smoke-exposed groups. This finding is considered to be caused by general stress associated with exposure treatment and smoke irritation (Gruver and Sempowski, 2008) and was also reflected in a decrease of the weight of the thymus relative to the body weight (Table 6). In general, the magnitude of the effect was more pronounced in the reference cigarette 2R4F smoke-exposed rats than in Kretek-R or Garam cigarette smoke-exposed rats. However, relative to sham, adrenal absolute weights were increased in a TPM-concentration-dependent way, but the changes were similar upon exposure to the smoke from the different cigarettes. Other findings occasionally reached statistical significance but did not follow a dose response and have not been consistently observed in other inhalation studies. They are therefore considered incidental.

3.11. Histopathology of the respiratory tract

Histopathological findings consistent with previous inhalation studies of cigarette smoke were observed in the smoke-exposed groups. These include histopathological changes in the nose (hyperplasia and squamous metaplasia of the respiratory epithelium, squamous metaplasia and atrophy of the olfactory epithelium), larynx (hyperplasia of the squamous epithelium and squamous metaplasia of the pseudostratified epithelium), and lungs (accumulation of macrophages and goblet cell hyperplasia) as detailed in Tables A–D in the Appendix. The histological changes observed in the larynx, lungs and trachea are also reflected in the changes of the weights (relative to body weight) of these organs (Table 6).

Significant differences in histopathological findings were observed between animals exposed to smoke from Garam or

Table 6
Organ weights, relative to body weight, after 90-day inhalation exposure to the MS of reference cigarette 2R4F, Garam and Kretek-R cigarettes.

Group	Lungs with Trachea and Larynx ($\times 10^{-4}$)		Thymus ($\times 10^{-4}$)	
	Male	Female	Male	Female
Study A				
Sham	35.09 ± 0.53	46.71 ± 1.02	5.76 ± 0.49	10.39 ± 1.02
2R4F Low	40.49 ± 1.19***	55.20 ± 0.80***	5.22 ± 54	8.63 ± 0.96
2R4F Medium	43.07 ± 0.67***	60.85 ± 0.97***	5.03 ± 0.39	6.51 ± 0.49
2R4F High	46.05 ± 1.27***	59.35 ± 1.21***	4.93 ± 0.50	6.61 ± 0.64
Garam Low	39.64 ± 0.93**	50.88 ± 1.06*	5.27 ± 0.38	8.52 ± 0.44
Garam Medium	40.65 ± 0.84***	55.43 ± 0.93***	5.07 ± 0.39	7.94 ± 0.54
Garam High	42.65 ± 0.84***	54.73 ± 0.85***	4.54 ± 0.46	10.24 ± 2.98
Study B				
Sham	36.25 ± 0.74	48.27 ± 0.63	4.65 ± 0.44	7.24 ± 0.50
2R4F Low	44.23 ± 0.95***	55.96 ± 1.40***	4.53 ± 0.41	5.20 ± 0.32*
2R4F Medium	47.36 ± 2.43***	60.46 ± 2.11***	3.22 ± 0.28	4.47 ± 0.50***
2R4F High	51.77 ± 0.73***	64.90 ± 1.41***	2.83 ± 0.33**	3.77 ± 0.32***
Kretek-R Low	40.81 ± 0.92	52.20 ± 1.45	4.41 ± 0.39	6.30 ± 0.61
Kretek-R Medium	43.43 ± 1.01**	55.53 ± 1.34**	3.91 ± 0.42	5.22 ± 0.47*
Kretek-R High	45.77 ± 1.06***	57.86 ± 1.39***	3.47 ± 0.20	4.42 ± 0.40***

Results are presented as mean ± standard error.
Difference from sham; significance: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).
Abbreviations: Garam, *Gudang Garam International Filter*; Kretek-R, *kretek reference cigarette.*

Kretek-R cigarettes and animals exposed to comparable levels of TPM from the reference cigarette 2R4F. The histopathological results were qualitatively similar but were generally less severe in animals exposed to smoke from Garam and Kretek-R cigarettes than in those exposed to smoke from the reference cigarette 2R4F. While differences between the reference cigarette 2R4F and Kretek-R cigarette smoke-exposed groups reached statistical significance in both male and female rats, the difference between Garam cigarette and the reference cigarette 2R4F smoke-exposed groups was only significant in female animals. No difference was observed on the effects on the epithelia of the male rats when comparing the reference cigarette 2R4F and the Garam cigarette smoke-exposed groups. This is probably due to the fact that, in general, all effects observed in the epithelia of the reference cigarette 2R4F-exposed male rats during the study of the Kretek-R cigarette were unexpectedly low (in some cases absent). Tables 7 and 8 list the observations which were significantly different between animals of either gender exposed to Garam or Kretek-R cigarette smoke and the corresponding reference cigarette 2R4F groups. Most histopathological findings had reversed (completely or partially) at the end of the 42-day recovery period.

4. Discussion

The marketed kretek cigarette with the highest TPM delivery (Garam) and an experimental kretek reference cigarette (Kretek-R) were tested in parallel with the American-blended reference cigarette 2R4F in two separate 90-day inhalation studies on rats. The exposure was assessed by monitoring the levels of different smoke constituents in the exposure chamber and the levels of some of their respective urinary biomarkers. Similar urinary levels of nicotine metabolites were observed in kretek- and the reference cigarette 2R4F-exposed animals when adjusted for the test atmosphere nicotine concentration. These results suggests similar smoke uptake by the animals occurred for both cigarette types, and this was further corroborated by similarity in the respiratory function parameters across all smoke-exposed groups. Furthermore, the measurements of metabolites of gas phase smoke constituents (CO, acrolein and 1,3-butadiene) were consistent with increased TPM exposure.

Inhalation studies have been performed with various animal species to assess cigarette smoke toxicity, as detailed in a review (Coggins, 2010). Many of the 'smoke-related' observations made from assaying both the kretek cigarettes and the American-blended reference cigarette 2R4F in the present study were similar to those reported from several MS inhalation studies in rats (Baker et al., 2004; Coggins et al., 2011; Roemer et al., 2012; Terpstra et al., 2003; Vanscheeuwijck et al., 2002). However, there are notable differences between kretek cigarette smoke-exposed groups and the groups exposed to smoke from the reference cigarette 2R4F. At equivalent TPM concentrations in the test atmosphere, the severity of almost all observed adverse effects was lower in groups exposed to smoke from both Garam and Kretek-R cigarettes than in those exposed to the reference cigarette 2R4F. In particular, both kretek cigarettes demonstrated less severity of most histopathological endpoints in the respiratory tract and a pronounced attenuation in pulmonary inflammation when compared to the reference cigarette 2R4F.

One exception to this general pattern of histopathological findings was observed in the context of Study B (Kretek-R cigarettes). It comes as a consequence of the unexpected low response to the smoke from the reference cigarette 2R4F observed on the respiratory tract organs in the male rats (and not the female animals). The 2R4F cigarette was used as a reference in many different inhalation studies, and no such effect was ever observed in these other studies; notably no such effect was observed when testing the reference cigarette 2R4F in Study A. Therefore, this observation cannot be considered reliable, whilst no obvious cause for this effect could be identified.

Lung inflammation was lower in kretek cigarette smoke-exposed than in the reference cigarette 2R4F-exposed rats, as evidenced by the lower number of neutrophils recovered from BALF (absolute numbers and% of total free lung cells) as well as the lower histopathological scores on the presence of alveolar macrophages. This is corroborated by the neutrophil counts in the blood. These effects were similar to those reported for an Electrically Heated Cigarette Smoking System (EHCSS) by Werley and his team at the same range of TPM exposures (100, 150 and 200 μg/l) (Werley et al., 2008).

Previous MS inhalation studies have demonstrated that exposure to the isolated gas/vapor phase (GVP) is mainly associated with histopathological changes in the nose of rats (Friedrichs et al., 2006), which likely reflects the presence of known irritants in the GVP (van der Toorn et al., 2013). The significant reduction in the majority of analyzed GVP constituents in kretek cigarettes relative to 2R4F cigarettes (Piadé et al., 2014) is therefore likely to explain at

Table 7
Histopathological endpoints statistically significantly different between reference cigarette 2R4F- and Garam cigarette smoke-exposed rats (Study A).

Localization	Tissue type	Observation	Garam vs. 2R4F	
			Male	Female
Nose level 1	Respiratory epithelium	Goblet cell hyperplasia	=	*↑
	Lumen	Exudate	*↑	=
Nose level 2	Respiratory epithelium	Reserve cell hyperplasia	=	***↓
		Squamous metaplasia without cornification	=	***↓
	Olfactory epithelium	Atrophy	=	***↓
		Squamous metaplasia without cornification	=	***↓
	Olfactory region	Loss of nerve bundles	=	***↓
Nose level 3	Olfactory epithelium	Atrophy	=	**↓
		Squamous metaplasia without cornification	=	**↓
	Olfactory region	Loss of nerve bundles	=	**↓
Nose level 4	Olfactory epithelium	Atrophy	=	**↓
		Squamous metaplasia without cornification	=	*↓
Larynx	Ventral depression	Squamous metaplasia without cornification	=	*↓
	Vocal cords, Upper medial region	Squamous metaplasia without cornification	***↓	=
	Pseudostratified epithelium	Squamous metaplasia without cornification	=	*↓
Tracheal bifurcation	Respiratory epithelium	Goblet cell hyperplasia	=	*↓
Left lung	Respiratory epithelium	Goblet cell hyperplasia	=	*↓
	Alveoli	Alveolar macrophages without pigmentation	=	**↓
		Alveolar macrophages with pigmentation	***↓	***↓

Difference among cigarettes; significance: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).
↑ indicates response higher in Garam relative to the reference cigarette 2R4F.
↓ indicates response lower in Garam relative to the reference cigarette 2R4F.

least in part the lower severity of the nasal histopathological findings in rats exposed to the kretek cigarette smoke. Conversely, it was also reported (Friedrichs et al., 2006) that the particulate phase of smoke is mostly responsible for the inflammation of the lung and the hyperplastic and metaplastic epithelial changes in the larynx. In addition to the effects caused by the different particulate phase composition of the smoke from kretek and 2R4F cigarettes (Piadé et al., 2014), the specific chemical and pharmacological effects of eugenol could also contribute to the lower severity of end points in the larynx and the lungs of kretek cigarette smoke-exposed rats. A separate set of studies (Parts 4 and 5 in the current publication series) was therefore performed to discern the contributions from clove, clove components and tobacco type to biological activity *in vitro* and *in vivo* (Roemer et al., 2014a, c).

As detailed in the lead publication (Roemer et al., 2014b), one acute and two sub-acute animal inhalation studies performed in the 80ies yielded differing results regarding the *in vivo* toxicity of kretek cigarettes smoke (Clark, 1989, 1990; Lavoie et al., 1986). The present studies, together with the other 90-day inhalation studies performed according to the OECD guideline 413 (OECD, 1981) reported in this special supplement, may be used as scientific evidence to characterize the hazard potential of kretek cigarettes. Overall, the observations from the inhalation studies are consistent with the observations made on smoke chemistry

Table 8
Histopathological endpoints statistically significantly different between reference cigarette 2R4F- and Kretek-R-cigarette smoke exposed rats (Study B).

Localization	Tissue type	Observation	Kretek-R vs. 2R4F	
			Male	Female
Nose level 1	Respiratory epithelium	Reserve cell hyperplasia	*↓	=
		Squamous metaplasia with cornification	**↓	*↓
		Squamous metaplasia without cornification	**↓	=
		Loss of goblet cells	*↓	**↓
Nose level 2	Respiratory epithelium	Reserve cell hyperplasia	***↓	***↓
		Squamous metaplasia without cornification	*↓	*↓
	Olfactory epithelium	Atrophy	***↓	***↓
		Squamous metaplasia without cornification	***↓	***↓
	Olfactory region	Loss of nerve bundles	***↓	***↓
Nose level 3	Olfactory epithelium	Atrophy	**↓	***↓
		Squamous metaplasia without cornification	**↓	***↓
	Olfactory region	Loss of nerve bundles	**↓	***↓
Nose level 4	Olfactory epithelium	Atrophy	**↓	***↓
		Squamous metaplasia without cornification	**↓	**↓
	Olfactory region	Loss of nerve bundles	**↓	***↓
Larynx	Vocal cords, Lower medial region	Squamous hyperplasia without cornification	=	*↑
Left lung	Respiratory epithelium	Goblet cell hyperplasia	=	*↓
	Alveoli	Alveolar macrophages without pigmentation	***↓	***↓
		Alveolar macrophages with pigmentation	***↓	***↓

Difference among cigarettes; significance: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).
↑ indicates response higher in Kretek-R relative to the reference cigarette 2R4F.
↓ indicates response lower in Kretek-R relative to the reference cigarette 2R4F.

Table A
Histopathological findings in the respiratory tract of male rats after the 90-day inhalation period to MS of the reference cigarette 2R4F, Garam cigarette, or to air (sham), Study A, (mean score ± standard error and incidence).

Localization	Tissue type	Observation	Sham	2R4F Low	2R4F Medium	2R4F High	Garam Low	Garam Medium	Garam High
Nose level 1	Respiratory epithelium	Reserve cell hyperplasia	0.0 ± 0.0 0/10	2.0 ± 0.4 9/10***	2.9 ± 0.41 0/10***	2.8 ± 0.4 9/10***	2.3 ± 0.5 8/10***	1.6 ± 0.5 8/10***	3.1 ± 0.4 10/10***
		Goblet cell hyperplasia	0.1 ± 0.1 1/10	0.6 ± 0.2 5/10	0.6 ± 0.2 6/10*	1.4 ± 0.4 8/10**	0.6 ± 0.2 5/10	0.6 ± 0.3 3/10	1.2 ± 0.3 7/10**
		Loss of goblet cells	0.3 ± 0.2 3/10	1.1 ± 0.2 9/10**	2.9 ± 0.3 10/10***	1.7 ± 0.3 10/10***	1.2 ± 0.2 9/10**	1.6 ± 0.4 9/10**	2.4 ± 0.4 10/10***
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	0.3 ± 0.2 3/10	0.5 ± 0.2 4/10	0.3 ± 0.2 3/10	0.8 ± 0.4 4/10	0.2 ± 0.1 2/10	0.7 ± 0.3 5/10
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	1.8 ± 0.6 6/10**	2.5 ± 0.5 9/10***	1.8 ± 0.5 7/10**	2.1 ± 0.6 6/10**	1.3 ± 0.5 5/10*	2.8 ± 0.4 9/10***
	Lamina propria	Inflammatory cell infiltration	0.0 ± 0.0 0/10	1.1 ± 0.3 7/10**	1.2 ± 0.3 7/10**	1.2 ± 0.3 7/10**	1.5 ± 0.4 7/10**	1.1 ± 0.5 4/10*	1.9 ± 0.3 9/10***
	Lumen	Exudate	0.0 ± 0.0 0/10	0.2 ± 0.2 1/10	0.3 ± 0.2 2/10	0.3 ± 0.3 1/10	0.6 ± 0.4 2/10	0.9 ± 0.4 4/10	1.0 ± 0.4 5/10
Nose Level 2	Respiratory epithelium	Reserve cell hyperplasia	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.1 ± 0.11/10	0.1 ± 0.1 1/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
	Olfactory epithelium	Atrophy	0.3 ± 0.2 3/10	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10	0.7 ± 0.3 4/10	0.0 ± 0.0 0/9	0.0 ± 0.0 0/10	0.3 ± 0.2 3/10
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.2 ± 0.1 2/10	0.0 ± 0.0 0/9	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
	Olfactory region	Loss of nerve bundles	0.3 ± 0.2 3/10	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10	0.6 ± 0.3 3/10	0.0 ± 0.0 0/9	0.0 ± 0.0 0/10	0.3 ± 0.2 3/10
Nose Level 3	Olfactory epithelium	Atrophy	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
Nose Level 4	Olfactory epithelium	Atrophy	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
Larynx	Ventral depression	Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.6 ± 0.3 4/10	0.6 ± 0.3 3/9	1.0 ± 0.4 5/10	0.7 ± 0.3 4/10	0.6 ± 0.3 3/10	1.0 ± 0.4 5/10
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/9	0.1 ± 0.1 1/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10
	Vocal cords, lower medial region	Hyperplasia without cornification	0.3 ± 0.2 3/10	1.7 ± 0.2 10/10***	2.0 ± 0.0 9/9***	2.4 ± 0.3 10/10***	2.0 ± 0.1 10/10***	1.6 ± 0.2 10/10***	1.9 ± 0.2 10/10***
		Hyperplasia with cornification	0.0 ± 0.0 0/10	1.1 ± 0.6 3/10	0.9 ± 0.4 4/10*	3.4 ± 0.6 9/10***	1.5 ± 0.5 6/10**	0.6 ± 0.3 3/10	1.7 ± 0.7 5/10*
	Vocal cords, upper medial region	Hyperplasia	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/9	0.0 ± 0.0 0/10	0.3 ± 0.2 2/10	0.1 ± 0.1 1/10	0.0 ± 0.0 0/10
		Squamous metaplasia without cornification	0.5 ± 0.3 3/10	2.3 ± 0.5 10/10**	2.7 ± 0.3 9/9***	2.7 ± 0.5 10/10**	1.2 ± 0.4 8/10*	0.8 ± 0.3 5/8	1.9 ± 0.5 9/9**
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	1.0 ± 0.7 2/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/8	0.0 ± 0.0 0/9
	Pseudo-stratified epithelium	Squamous metaplasia without cornification	0.0 ± 0.0 0/10	3.8 ± 0.5 10/10***	4.7 ± 0.2 9/9***	4.8 ± 0.1 10/10***	3.0 ± 0.6 8/10***	4.2 ± 0.4 10/10***	4.7 ± 0.2 10/10***
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	2.1 ± 0.6 7/10**	2.4 ± 0.7 7/9***	3.5 ± 0.6 10/10***	1.7 ± 0.5 8/10***	2.0 ± 0.5 8/10***	4.0 ± 0.5 10/10***

Vocal folds	Squamous epithelium	Hyperplasia without cornification	0.6 ± 0.2 5/10	1.3 ± 0.2 10/10	1.2 ± 0.1 9/9	1.6 ± 0.3 9/10	1.3 ± 0.2 9/10	0.9 ± 0.3 6/10	1.4 ± 0.2 10/10
		Hyperplasia with cornification	0.1 ± 0.1 1/10	1.1 ± 0.4 5/10*	1.4 ± 0.5 5/9*	2.5 ± 0.5 8/10**	0.8 ± 0.3 5/10*	0.9 ± 0.3 5/10*	1.0 ± 0.3 6/10*
Tracheal bifurcation	Respiratory epithelium	Goblet cell hyperplasia	0.1 ± 0.1 1/10	0.1 ± 0.1 1/10	0.2 ± 0.1 2/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.2 ± 0.2 1/10	0.1 ± 0.1 1/10
		Squamous metaplasia without cornification	0.1 ± 0.1 1/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
Left lung	Respiratory epithelium	Goblet cell hyperplasia	0.6 ± 0.3 3/10	1.0 ± 0.6 4/8	0.7 ± 0.2 6/9	2.5 ± 0.6 8/10	1.0 ± 0.4 6/10	0.3 ± 0.2 2/8	1.4 ± 0.5 7/10
	Lamina propria	Inflammatory cell infiltration	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10	0.2 ± 0.1 2/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.1 ± 0.1 (1/10)
	Alveoli	Alveolar macrophages without pigmentation	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10	0.2 ± 0.1 2/10	0.5 ± 0.2 4/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/9	0.2 ± 0.1 2/10
		Alveolar macrophages with pigmentation	0.0 ± 0.0 0/10	0.2 ± 0.1 2/10	0.7 ± 0.2 7/10**	1.1 ± 0.2 9/10***	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.4 ± 0.2 4/10*

Difference from sham; significance: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).

Table B
Histopathological findings in the respiratory tract of female rats after the 90-day inhalation period to MS of the reference cigarette 2R4F, Garam cigarette, or to air (sham), Study A, (mean score ± standard error and incidence).

Localization	Tissue type	Observation	Sham	2R4F Low	2R4F Medium	2R4F High	Garam Low	Garam Medium	Garam High
Nose Level 1	Respiratory epithelium	Reserve cell hyperplasia	0.0 ± 0.0 0/10	2.1 ± 0.4 9/10***	2.4 ± 0.3 10/10***	3.0 ± 0.4 10/10***	1.5 ± 0.4 8/10***	2.7 ± 0.4 10/10***	2.7 ± 0.4 9/10***
		Goblet cell hyperplasia	0.0 ± 0.0 0/10	0.6 ± 0.3 4/10*	1.0 ± 0.2 8/10***	0.6 ± 0.2 5/10*	1.3 ± 0.4 6/10**	1.4 ± 0.5 7/10**	1.4 ± 0.3 8/10***
		Loss of goblet cells	0.3 ± 0.2 3/10	2.1 ± 0.3 10/10***	2.9 ± 0.3 10/10***	3.0 ± 0.4 10/10***	1.4 ± 0.2 10/10***	2.4 ± 0.3 10/10***	2.6 ± 0.3 10/10***
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	0.7 ± 0.3 4/10*	0.9 ± 0.2 7/10**	0.8 ± 0.2 7/10**	0.9 ± 0.4 5/10*	1.0 ± 0.4 7/10**	1.1 ± 0.3 8/10***
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	2.5 ± 0.5 9/10***	3.3 ± 0.4 10/10***	3.4 ± 0.4 9/10***	2.6 ± 0.5 9/10***	3.0 ± 0.5 9/10***	3.4 ± 0.3 10/10***
	Lamina propria	Inflammatory cell infiltration	0.1 ± 0.1 1/10	1.7 ± 0.5 7/10**	1.7 ± 0.3 9/10***	1.9 ± 0.3 10/10***	0.8 ± 0.2 6/10*	2.4 ± 0.4 9/10***	2.2 ± 0.4 10/10***
	Lumen	Exudate	0.0 ± 0.0 0/10	1.0 ± 0.5 3/10	0.7 ± 0.4 3/10	1.1 ± 0.4 5/10*	0.1 ± 0.1 1/10	2.0 ± 0.5 7/10**	1.4 ± 0.5 7/10**
Nose Level 2	Respiratory epithelium	Reserve cell hyperplasia	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.5 ± 0.2 5/10*	0.7 ± 0.3 5/10*	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
		Squamous metaplasia without cornification	0.6 ± 0.2 6/10	0.2 ± 0.1 2/10	0.8 ± 0.1 8/10	0.9 ± 0.2 8/10	0.1 ± 0.1 1/10*	0.3 ± 0.2 3/10	0.2 ± 0.1 2/10
	Olfactory epithelium	Atrophy	0.1 ± 0.1 1/10	0.2 ± 0.1 2/10	2.0 ± 0.6 6/10*	3.0 ± 0.6 9/10***	0.0 ± 0.0 0/10	0.2 ± 0.1 2/10	0.2 ± 0.1 2/10
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.9 ± 0.4 4/10*	2.1 ± 0.6 7/10**	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10

(*continued on next page*)

Table B (*continued*)

Localization	Tissue type	Observation	Sham	2R4F Low	2R4F Medium	2R4F High	Garam Low	Garam Medium	Garam High
	Olfactory region	Loss of nerve bundles	0.1 ± 0.1 1/10	0.2 ± 0.1 2/10	1.7 ± 0.7 5/10*	3.0 ± 0.6 9/10***	0.0 ± 0.0 0/10	0.2 ± 0.1 2/10	0.2 ± 0.1 2/10
Nose Level 3	Olfactory epithelium	Atrophy	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.7 ± 0.4 3/10	1.8 ± 0.6 5/10*	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.4 ± 0.3 2/10	0.8 ± 0.3 4/10*	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.7 ± 0.4 3/10	1.8 ± 0.6 5/10*	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
Nose Level 4	Olfactory epithelium	Atrophy	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.6 ± 0.4 2/10	1.6 ± 0.7 4/10*	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.4 ± 0.4 1/10	1.4 ± 0.6 4/10*	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.2 ± 0.1 1/10	0.4 ± 0.4 1/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
Larynx	Ventral depression	Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.6 ± 0.3 4/10*	2.3 ± 0.5 9/10***	2.3 ± 0.4 10/10***	1.0 ± 0.4 6/10**	0.6 ± 0.2 6/10**	1.3 ± 0.4 8/10***
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.3 ± 0.2 2/10	0.4 ± 0.2 3/10	0.3 ± 0.3 1/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
	Vocal cords, lower medial region	Hyperplasia without cornification	0.6 ± 0.2 5/10	2.2 ± 0.2 10/10***	2.7 ± 0.3 10/10***	3.0 ± 0.3 10/10***	2.1 ± 0.2 10/10***	2.8 ± 0.2 10/10***	2.3 ± 0.2 10/10***
		Hyperplasia with cornification	0.0 ± 0.0 0/10	1.6 ± 0.6 5/10*	3.7 ± 0.7 8/10***	2.5 ± 0.8 6/10**	0.3 ± 0.2 2/10**	4.2 ± 0.5 9/10***	3.6 ± 0.7 8/10***
	Vocal cords, upper medial region	Squamous metaplasia without cornification	0.4 ± 0.3 2/7	2.0 ± 0.5 9/9	3.1 ± 0.6 10/10	3.0 ± 0.4 9/9	2.4 ± 0.6 8/8	3.4 ± 0.6 10/10	3.4 ± 0.6 9/9
		Squamous metaplasia with cornification	0.0 ± 0.0 0/7	1.1 ± 0.7 2/9	1.9 ± 0.8 4/10	0.6 ± 0.5 2/10	0.0 ± 0.0 0/8	2.4 ± 0.8 5/10	2.2 ± 0.9 4/9
	Pseudo-stratified epithelium	Squamous metaplasia without cornification	0.1 ± 0.1 1/10	5.0 ± 0.0 10/10***	5.0 ± 0.0 10/10***	5.0 ± 0.0 10/10***	4.6 ± 0.2 10/10***	4.9 ± 0.1 10/10***	5.0 ± 0.0 10/10***
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	4.2 ± 0.3 10/10***	4.7 ± 0.2 10/10***	4.8 ± 0.1 10/10***	2.9 ± 0.5 9/10***	4.2 ± 0.3 10/10***	4.9 ± 0.1 10/10***
Vocal folds	Squamous epithelium	Hyperplasia without cornification	0.1 ± 0.1 1/10	1.7 ± 0.2 10/10***	1.6 ± 0.2 9/10***	1.5 ± 0.2 9/10***	1.5 ± 0.2 9/10***	1.7 ± 0.2 10/10***	1.8 ± 0.1 10/10***
		Hyperplasia with cornification	0.0 ± 0.0 0/10	2.1 ± 0.4 8/10***	3.1 ± 0.7 9/10***	2.1 ± 0.7 5/10*	1.4 ± 0.4 6/10**	2.7 ± 0.6 8/10***	3.4 ± 0.6 9/10***
Tracheal bifurcation	Respiratory epithelium	Goblet cell hyperplasia	0.1 ± 0.1 1/10	0.0 ± 0.0 0/10	0.2 ± 0.1 2/10	0.4 ± 0.2 3/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
		Squamous metaplasia without cornification	0.3 ± 0.2 3/10	0.1 ± 0.1 1/10	0.3 ± 0.2 3/10	0.1 ± 0.1 1/10	0.2 ± 0.1 2/10	0.1 ± 0.1 1/10	0.3 ± 0.2 2/10
Left lung	Respiratory epithelium	Goblet cell hyperplasia	0.9 ± 0.4 5/10	2.0 ± 0.6 8/10	2.1 ± 0.6 8/9*	3.4 ± 0.5 10/10**	0.7 ± 0.3 4/10**	0.8 ± 0.4 4/10	3.2 ± 0.5 9/9**
	Lamina propria	Inflammatory cell infiltration	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/9	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10
	Alveoli	Alveolar macrophages without pigmentation	0.2 ± 0.1 2/10	0.6 ± 0.2 6/10	0.8 ± 0.1 7/9**	0.9 ± 0.1 9/10**	0.2 ± 0.1 2/10	0.5 ± 0.2 5/10	0.6 ± 0.2 6/10
		Alveolar macrophages with pigmentation	0.0 ± 0.0 0/10	0.6 ± 0.2 6/10**	1.4 ± 0.2 9/9***	1.5 ± 0.2 10/10***	0.3 ± 0.2 3/10	0.7 ± 0.2 6/10**	0.5 ± 0.2 5/10*

Difference from Sham; significance: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).

Table C
Histopathological findings in the respiratory tract of male rats after the 90-day inhalation period to MS of the reference cigarette 2R4F,Kretek-R cigarette, or to air (sham), Study B, (mean score ± standard error and incidence).

Localization	Tissue type	Observation	Sham	2R4F low	2R4F Medium	2R4F High	Kretek-R Low	Kretek-R Medium	Kretek-R High
Nose Level 1	Respiratory epithelium	Reserve cell hyperplasia	0.1 ± 0.1	3.0 ± 0.1	4.0 ± 0.0	3.9 ± 0.1	2.7 ± 0.3	3.4 ± 0.2	3.7 ± 0.2
			1/10	10/10***	9/9***	9/9***	10/10***	10/10***	9/9***
		Goblet cell hyperplasia	0.0 ± 0.0	0.3 ± 0.3	1.0 ± 0.3	0.9 ± 0.3	0.9 ± 0.4	1.5 ± 0.3	0.9 ± 0.3
			0/10	2/10	6/9**	6/9**	6/10**	8/10***	6/9**
		Loss of goblet cells	0.2 ± 0.1	2.7 ± 0.3	3.4 ± 0.2	4.0 ± 0.2	2.5 ± 0.3	3.0 ± 0.3	3.0 ± 0.3
			2/10	10/10***	9/9***	9/9***	10/10***	10/10***	9/9***
		Squamous metaplasia with cornification	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.3	1.9 ± 0.4	0.0 ± 0.0	0.5 ± 0.3	0.0 ± 0.0
			0/10	0/10	4/9*	7/9***	0/10	2/10	0/9
		Squamous metaplasia without cornification	0.0 ± 0.0	1.7 ± 0.2	3.2 ± 0.3	3.7 ± 0.2	1.5 ± 0.4	2.3 ± 0.4	2.4 ± 0.3
			0/10	10/10***	9/9***	9/9***	7/10**	10/10***	9/9***
Nose Level 2	Respiratory epithelium	Reserve cell hyperplasia	0.2 ± 0.1	1.3 ± 0.2	2.8 ± 0.2	3.7 ± 0.2	1.0 ± 0.2	1.4 ± 0.2	1.6 ± 0.2
			2/10	10/10***	10/10***	9/9***	8/10**	10/10***	9/9***
		Squamous metaplasia without cornification	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/10	0/10	0/10	4/9*	0/10	0/10	0/9
	Olfactory epithelium	Atrophy	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.5	2.8 ± 0.4	0.2 ± 0.1	0.0 ± 0.0	0.8 ± 0.3
			0/9	0/10	8/10***	9/9***	2/10	0/10	5/9*
		Squamous metaplasia without cornification	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.4	1.4 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/9	0/10	4/10*	5/9*	0/10	0/10	0/9
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.3	2.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
			0/9	0/10	4/10*	7/9**	0/10	0/10	2/9
Nose Level 3	Olfactory epithelium	Atrophy	0.0 ± 0.0	0.1 ± 0.1	1.5 ± 0.6	1.8 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/10	1/10	5/10*	4/9*	0/10	0/10	0/9
		Squamous metaplasia without cornification	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.3	1.4 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/10	0/10	4/10*	4/9*	0/10	0/10	0/9
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.3	1.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/10	0/10	4/10*	4/9*	0/10	0/10	0/9
Nose Level 4	Olfactory epithelium	Atrophy	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.4	1.2 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/9	0/10	3/10	4/9*	0/10	0/10	0/9
		Squamous metaplasia without cornification	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3	1.2 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/9	0/10	2/10	4/9*	0/10	0/10	0/9
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	1.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/9	0/10	3/10	4/9*	0/10	0/10	0/9
Larynx	Ventral depression	Squamous metaplasia without cornification	0.0 ± 0.0	1.1 ± 0.2	2.3 ± 0.4	3.4 ± 0.5	1.6 ± 0.5	1.9 ± 0.6	2.1 ± 0.5
			0/10	8/10***	9/10***	9/9***	8/9***	7/10**	7/9***
		Squamous metaplasia with cornification	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.7	0.2 ± 0.2	0.5 ± 0.5	0.6 ± 0.6
			0/10	0/10	0/10	3/9	1/9	1/10	1/9
	Vocal cords, LMR	Hyperplasia without cornification	0.0 ± 0.0	2.6 ± 0.3	3.6 ± 0.2	4.0 ± 0.0	3.2 ± 0.3	3.6 ± 0.2	3.8 ± 0.1
			0/10	10/10***	10/10***	9/9***	9/9***	8/8***	9/9***
		Hyperplasia with cornification	0.0 ± 0.0	0.7 ± 0.5	2.3 ± 0.5	3.1 ± 0.5	2.3 ± 0.6	1.9 ± 0.7	3.3 ± 0.5
			0/10	2/10	7/10**	8/8***	7/9***	5/9**	8/9***
	Vocal cords, UMR	Squamous metaplasia without cornification	0.0 ± 0.0	1.4 ± 0.5	2.7 ± 0.5	3.9 ± 0.1	1.4 ± 0.5	2.6 ± 0.4	3.4 ± 0.3
			0/10	7/10**	10/10***	7/7***	5/9**	9/9***	9/9***
	Pseudo-stratified epithelium	Squamous metaplasia without cornification	0.0 ± 0.0	4.7 ± 0.2	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	4.9 ± 0.1	4.9 ± 0.1
			0/10	10/10***	10/10***	9/9***	9/9***	10/10***	9/9***
		Squamous metaplasia with cornification	0.0 ± 0.0	3.8 ± 0.4	4.6 ± 0.2	4.9 ± 0.1	4.7 ± 0.2	4.5 ± 0.3	4.9 ± 0.1
			0/10	10/10***	10/10***	9/9***	9/9***	10/10***	9/9***
Vocal folds	Pseudo-stratified epithelium	Reserve cell hyperplasia	0.0 ± 0.0	0.8 ± 0.2	1.2 ± 0.2	1.9 ± 0.3	0.8 ± 0.3	1.4 ± 0.3	1.4 ± 0.4
			0/10	6/10**	9/10***	7/7***	4/8*	7/8***	6/7***
		Squamous metaplasia without cornification	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/10	0/10	0/10	1/7	0/8	0/8	0/7
	Squamous epithelium	Hyperplasia without cornification	0.0 ± 0.0	1.3 ± 0.4	2.7 ± 0.4	3.3 ± 0.3	2.1 ± 0.2	2.6 ± 0.3	3.4 ± 0.2
			0/10	6/10**	9/10***	7/7***	8/8***	8/8***	7/4***
		Hyperplasia with cornification	0.0 ± 0.0	0.9 ± 0.4	2.8 ± 0.4	4.0 ± 0.4	2.1 ± 0.4	2.8 ± 0.5	3.9 ± 0.3

(*continued on next page*)

Table C (*continued*)

Localization	Tissue type	Observation	Sham	2R4F low	2R4F Medium	2R4F High	Kretek-R Low	Kretek-R Medium	Kretek-R High
			0/10	4/10*	9/10***	7/7***	7/8***	7/8***	7/7***
Tracheal bifurcation	Respiratory epithelium	Goblet cell hyperplasia	0.4 ± 0.2	0.2 ± 0.1	1.0 ± 0.2	1.3 ± 0.3	0.2 ± 0.1	0.6 ± 0.2	0.8 ± 0.3
			4/10	2/9	8/9*	7/8*	2/9	5/9	4/8
		Squamous metaplasia without cornification	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
			0/9	0/8	0/8	0/6	0/8	1/9	1/7
Left lung	Respiratory epithelium	Goblet cell hyperplasia	0.7 ± 0.6	0.8 ± 0.2	2.3 ± 0.4	3.9 ± 0.4	0.8 ± 0.2	2.0 ± 0.4	2.7 ± 0.6
			2/9	5/6	9/10*	7/7**	6/10	10/10**	7/7**
	Alveoli	Alveolar macrophages without pigmentation	0.2 ± 0.1	1.4 ± 0.2	2.0 ± 0.0	2.8 ± 0.1	0.8 ± 0.1	1.4 ± 0.2	1.6 ± 0.2
			2/10	10/10***	10/10***	9/9***	8/10**	10/10***	9/9***
		Alveolar macrophages with pigmentation	0.0 ± 0.0	1.9 ± 0.2	2.7 ± 0.2	3.4 ± 0.2	1.0 ± 0.2	1.9 ± 0.1	2.0 ± 0.2
			0/10	10/10***	10/10***	9/9***	8/10***	10/10***	9/9***

Difference from Sham; Significance: $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).

Table D
Histopathological findings in the respiratory tract of female rats after the 90-day inhalation period to MS of the reference cigarette 2R4F, Kretek-R cigarette, or to air (sham), Study B, (mean score ± standard error and incidence).

Localization	Tissue type	Observation	Sham	2R4F Low	2R4F Medium	2R4F High	Kretek-R Low	Kretek-R Medium	Kretek-R High
Nose Level 1	Respiratory epithelium	Reserve cell hyperplasia	0.1 ± 0.1	3.3 ± 0.2	4.0 ± 0.0	4.0 ± 0.0	3.1 ± 0.2	3.7 ± 0.2	4.0 ± 0.0
			1/10	10/10***	10/10***	10/10***	10/10***	10/10***	9/9***
		Goblet cell hyperplasia	0.0 ± 0.0	0.3 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	1.1 ± 0.2
			0/10	3/10	7/10**	3/10*	4/10*	4/10*	8/9***
		Loss of goblet cells	0.3 ± 0.2	3.6 ± 0.2	4.4 ± 0.2	4.7 ± 0.2	3.2 ± 0.2	4.1 ± 0.2	4.1 ± 0.1
			3/10	10/10***	10/10***	10/10***	10/10***	10/10***	9/9***
		Squamous metaplasia with cornification	0.0 ± 0.0	0.1 ± 0.1	1.8 ± 0.4	2.4 ± 0.3	0.4 ± 0.3	0.9 ± 0.3	1.0 ± 0.4
			0/10	1/10	7/10**	9/10***	2/10	5/10*	4/9*
		Squamous metaplasia without cornification	0.0 ± 0.0	2.5 ± 0.3	3.7 ± 0.2	3.9 ± 0.1	2.4 ± 0.3	3.2 ± 0.3	3.6 ± 0.2
			0/10	10/10***	10/10***	10/10***	10/10***	10/10***	9/9***
Nose Level 2	Respiratory epithelium	Reserve cell hyperplasia	0.1 ± 0.1	1.4 ± 0.2	2.5 ± 0.2	3.6 ± 0.2	0.5 ± 0.2	0.9 ± 0.1	1.6 ± 0.2
			1/10	9/10***	10/10***	10/10***	5/10	9/10***	9/9***
		Squamous metaplasia without cornification	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
			0/10	0/10	1/10	3/10	0/10	0/10	0/9
	Olfactory epithelium	Atrophy	0.0 ± 0.0	0.5 ± 0.2	1.9 ± 0.5	3.5 ± 0.3	0.1 ± 0.1	0.0 ± 0.0	1.4 ± 0.3
			0/10	4/10*	7/10**	10/10***	1/10	0/10	7/9***
		Squamous metaplasia without cornification	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.4	1.9 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
			0/10	0/10	3/10	8/10***	0/10	0/10	1/9
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0	0.1 ± 0.1	0.9 ± 0.3	3.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.4
			0/10	1/10	5/10*	10/10***	0/10	0/10	5/9**
Nose Level 3	Olfactory epithelium	Atrophy	0.1 ± 0.1	0.3 ± 0.3	1.0 ± 0.5	3.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.4
			1/10	1/10	3/10	10/10***	0/9	0/10	3/9
		Squamous metaplasia without cornification	0.0 ± 0.0	0.1 ± 0.1	0.9 ± 0.5	2.4 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
			0/10	1/10	3/10	8/10***	0/10	0/10	1/9

	Olfactory region	Loss of nerve bundles	0.0 ± 0.0 0/10	0.2 ± 0.2 1/10	0.7 ± 0.4 3/10	2.9 ± 0.5 9/10***	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.6 ± 0.3 3/9
Nose Level 4	Olfactory epithelium	Atrophy	0.0 ± 0.0 0/10	0.2 ± 0.2 1/10	0.7 ± 0.4 3/10	2.3 ± 0.4 8/10***	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/9
		Squamous metaplasia without cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.3 ± 0.3 1/10	1.5 ± 0.5 6/10**	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/9
	Olfactory region	Loss of nerve bundles	0.0 ± 0.0 0/10	0.1 ± 0.1 1/10	0.4 ± 0.2 3/10	1.8 ± 0.5 7/10**	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.0 ± 0.0 0/9
Larynx	Ventral depression	Squamous metaplasia without cornification	0.0 ± 0.0 0/10	1.6 ± 0.5 8/10***	2.7 ± 0.5 8/9***	3.9 ± 0.4 10/10***	1.1 ± 0.2 8/9***	2.6 ± 0.4 9/9***	3.0 ± 0.4 9/9***
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	0.0 ± 0.0 0/10	0.6 ± 0.4 2/9	0.9 ± 0.5 3/10	0.0 ± 0.0 0/9	0.0 ± 0.0 0/9	0.3 ± 0.3 1/9
	Vocal cords, lower medial region	Hyperplasia without cornification	0.0 ± 0.0 0/9	3.7 ± 0.2 10/10***	3.8 ± 0.2 10/10***	3.9 ± 0.1 10/10***	3.9 ± 0.1 10/10***	4.0 ± 0.0 9/9***	4.3 ± 0.2 9/9***
		Hyperplasia with cornification	0.0 ± 0.0 0/9	3.0 ± 0.5 8/10***	3.7 ± 0.5 9/10***	4.1 ± 0.2 10/10***	3.8 ± 0.2 10/10***	4.3 ± 0.2 9/9***	4.0 ± 0.2 9/9***
	Vocal cords, upper medial region	Squamous metaplasia without cornification	0.0 ± 0.0 0/9	3.4 ± 0.5 9/10***	3.7 ± 0.6 9/10***	3.7 ± 0.4 9/9***	2.9 ± 0.5 9/9***	4.0 ± 0.7 7/7***	3.7 ± 0.4 9/9***
		Squamous metaplasia with cornification	0.0 ± 0.0 0/9	0.0 ± 0.0 0/10	1.2 ± 0.6 3/10	0.8 ± 0.6 2/9	0.3 ± 0.3 1/9	1.9 ± 0.9 3/7	1.1 ± 0.7 2/9
	Pseudo-stratified epithelium	Squamous metaplasia without cornification	0.2 ± 0.2 1/10	4.9 ± 0.1 10/10***	5.0 ± 0.0 10/10***	5.0 ± 0.0 10/10***	5.0 ± 0.0 10/10***	5.0 ± 0.0 10/10***	5.0 ± 0.0 9/9***
		Squamous metaplasia with cornification	0.0 ± 0.0 0/10	4.7 ± 0.2 10/10***	4.8 ± 0.2 10/10***	5.0 ± 0.0 10/10***	4.8 ± 0.2 10/10***	4.9 ± 0.2 10/10***	5.0 ± 0.0 9/9***
Vocal folds	Pseudo-stratified epithelium	Reserve cell hyperplasia	0.1 ± 0.1 1/10	0.3 ± 0.2 3/9	1.4 ± 0.21 0/10***	1.9 ± 0.2 10/10***	0.7 ± 0.2 5/9*	1.2 ± 0.2 9/10***	1.4 ± 0.3 7/8**
	Squamous epithelium	Hyperplasia without cornification	0.2 ± 0.1 2/10	2.2 ± 0.4 8/9**	2.9 ± 0.3 10/10***	3.7 ± 0.2 10/10***	2.7 ± 0.2 9/9***	3.1 ± 0.3 10/10***	3.3 ± 0.3 8/8***
		Hyperplasia with cornification	0.0 ± 0.0 0/10	1.9 ± 0.5 6/9**	3.1 ± 0.4 9/10***	4.1 ± 0.2 10/10***	2.7 ± 0.4 9/9***	3.2 ± 0.4 9/10***	3.3 ± 0.3 8/8***
Tracheal bifurcation	Respiratory epithelium	Goblet cell hyperplasia	0.8 ± 0.3 (5/9)	0.1 ± 0.1 1/10	0.7 ± 0.3 5/10	0.8 ± 0.1 8/10	0.3 ± 0.2 3/10	0.4 ± 0.2 3/10	0.8 ± 0.1 7/9
		Squamous metaplasia without cornification	0.0 ± 0.0 (0/8)	0.0 ± 0.0 0/10	0.1 ± 0.1 1/9	0.4 ± 0.2 3/9	0.1 ± 0.1 1/9	0.1 ± 0.1 1/10	0.0 ± 0.0 0/8
Left lung	Respiratory epithelium	Goblet cell hyperplasia	0.5 ± 0.3 3/8	0.9 ± 0.3 6/9	2.2 ± 0.4 10/10**	4.0 ± 0.3 8/8***	0.7 ± 0.2 6/9	1.5 ± 0.4 8/10	2.4 ± 0.4 7/7**
	Alveoli	Alveolar macrophages without pigmentation	0.3 ± 0.2 3/10	2.2 ± 0.1 10/10***	2.7 ± 0.2 10/10***	2.4 ± 0.2 10/10***	1.2 ± 0.1 10/10**	1.7 ± 0.2 10/10***	2.1 ± 0.2 9/9***
		Alveolar macrophages with pigmentation	0.2 ± 0.1 2/10	2.5 ± 0.3 10/10***	3.0 ± 0.11 0/10***	3.0 ± 0.1 10/10***	1.2 ± 0.1 10/10***	1.9 ± 0.2 10/10***	2.2 ± 0.1 9/9***

Difference from sham; Significance: $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ (p-value from 2-way ANOVA followed by Dunnett posthoc test).

and *in vitro* toxicology. They do not support any increased toxicity of the smoke of kretek cigarettes compared to conventional American-blended cigarettes.

5. Conclusions

Classical toxicology endpoints from two 90-day inhalation studies on two kretek cigarettes and an American-blended reference cigarette provided insight into their comparative toxicity, with the aim of providing a basis for hazard characterization. The observations were consistent with what has been observed in previously published MS inhalation studies in rodents. Nevertheless, kretek cigarette smoke exposure was associated with significantly lower pulmonary inflammation and histopathological changes in the respiratory tract. These results are consistent with the observations made on smoke chemistry and *in vitro* toxicology. They do not support any increased toxicity of the smoke of kretek cigarettes compared to conventional American-blended cigarettes.

Conflict of interest

All authors are or were Philip Morris International (PMI) R&D employees. The work reported here was funded by PMI R&D.

Acknowledgments

The authors are grateful to the staff at Hanjaya Mandala Sampoerna Tbk Indonesia who designed and manufactured the experimental cigarettes according to demanding specifications. Equally, they thank the involved staffs of Philip Morris Research Laboratories Cologne, Germany, and Leuven, Belgium for their excellent technical performance.

Appendix A.

See Tables A–D

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